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Resolution and Pharmacological Analysis of the Voltage-Dependent Calcium Channels of *Drosophila* Larval Muscles

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Voltage-dependent calcium channels play a role in many cellular phenomena. Very little is known about Ca^{2+} channels in *Drosophila*, especially those in muscles. Existing literature on neuronal Ca^{2+} channels of *Drosophila* suggests that their pharmacology may be distinct from that of vertebrate Ca^{2+} channels. This raises questions on the pharmacology and diversity of Ca^{2+} channels in *Drosophila* muscles. Here we show that the Ca^{2+} channel current in the body-wall muscles of *Drosophila* larvae consists of two main components. One component is sensitive to 1,4-dihydropyridines and diltiazem, which block vertebrate L-type Ca^{2+} channels. The second component is sensitive to amiloride, which blocks vertebrate T-type Ca^{2+} channels.

In contrast to *Drosophila* brain membrane preparations in which a majority of the Ca^{2+} channels are phenylalkylamine-sensitive but dihydropyridine-insensitive, the major current in the muscles was dihydropyridine-sensitive but relatively less sensitive to verapamil. This might indicate an underlying tissue specific distribution of distinct subtypes of dihydropyridine/phenylalkylamine-sensitive Ca^{2+} channels in *Drosophila*.

Low verapamil sensitivity of the dihydropyridine-sensitive current of *Drosophila* muscles also set it apart from the vertebrate L-type channels which are sensitive to 1,4-dihydropyridines, benzothiazepines as well as phenylalkylamines. The dihydropyridine-sensitive current in *Drosophila* muscles activated in a similar voltage range as the vertebrate L-type current. As with the vertebrate current, blockade by dihydropyridines was voltage dependent. Compared to the vertebrate T-type current, the amiloride-sensitive current in *Drosophila* muscles showed higher activation threshold as well as slower inactivation.

These experiments provide the first clear resolution of a *Drosophila* Ca^{2+} current into two distinct components. With the previous resolution of the K^+ current into four components, *Drosophila* larval muscles now provide one of the few preparations in which the whole cell current can be resolved completely into individual ionic currents. This will help in determining the role of individual currents in cellular excitability and other calcium related processes; in analyzing structure, function, and regulation of specific types of Ca^{2+} channels; as well as in understanding the molecular basis of calcium channel diversity.

[Key words:* Drosophila, *calcium currents, calcium channel blockers, dihydropyridines, verapamil, amiloride, pharmacology, physiology, muscle, larva, insects]

Voltage-dependent calcium channels have been observed in a variety of species. In addition to playing a functional role in many cellular processes (Augustine et al., 1985; Hille, 1992) they provide an entry for essential ions that serve as intracellular messengers. These channels are typically classified into several groups. L-Type channels have a high-voltage threshold for activation (Tsien et al., 1988; Bean, 1989) and are believed to be the dominating Ca^{2+} channel type in muscle preparations (Bean, 1989). Vertebrate L-type channels are blocked by 1,4-dihydropyridines (DHPs), phenylalkylamines and benzothiazepines, as the α_1 subunit contains a distinct but allosterically coupled receptor for each class of agents (for review, see Catterall et al., 1988; Hosey and Lazdunski, 1988; Glossmann and Striessnig, 1990; Triggle, 1990). T-type channels are characterized by transient, low voltage-activated currents (Fox et al., 1987) and have also been found in muscle preparations (Bean, 1985; Nilius et al., 1985; Cognard et al., 1986; Yatani et al., 1987). T-Type currents show sensitivity to octanol and flunarizine (Tytgat et al., 1988; Llinas et al., 1989), although these agents are nonselective. Selective T-channel blockade has been shown with amiloride (Tang et al., 1988; Hirano et al., 1989).

Leung and Byerly (1991) recorded single-channel Ca^{2+} currents from cultured *Drosophila* embryonic myotubes and neurons. Their studies suggest multiple types of calcium channels in neuronal cells, with fast, complicated kinetics. These channels are generally insensitive to the DHPs, phenylalkylamines, benzothiazepines, and ω-conotoxin GVIA (McCleskey et al., 1987; Byerly and Leung, 1988). They are instead blocked by spider toxins (Plectreurys toxin and Hololena toxin) (Leung et al., 1989). These data raise questions regarding whether calcium channels in *Drosophila*, and perhaps all insects, are insensitive to the classical calcium channel antagonists used for vertebrate systems. In addition, all Ca^{2+} channels in *Drosophila* neurons appear to have a similar voltage dependence of activation (Leung and Byerly, 1991). Comparable results are observed in another invertebrate system, as *Aplysia* bag neurons also possess different types of Ca^{2+} channel displaying a similar voltage dependence of activation (Strong et al., 1987).

Reconstitution studies with *Drosophila* head extracts show both a very high affinity and a low affinity site for phenylalkylamines (Pauron et al., 1987; Greenberg et al., 1989; Pelzer et al., 1989). Very little specific binding is detected for (+)-^{3}H-PN200-110 and *d-(cis)*-^{3}H-diltiazem (Greenberg et al., 1989).

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Phenylalkylamine binding is partially inhibited with diltiazem and is allosterically unaffected by high concentrations of 1,4-dihydropyridines. Pelzer et al. (1989) discovered Ca^{2+} channels in *Drosophila* brain membranes sensitive to phenylalkylamines alone and distinct channels sensitive to DHPs alone. These results indicate that the protein containing the phenylalkylamine receptor may be distinct from that containing the DHP receptor, giving Ca^{2+} channels from *Drosophila* brain membranes unique pharmacological properties.

Differences in pharmacological and physiological properties between Ca^{2+} channels from vertebrates and those from *Drosophila* and other invertebrates (Pauron et al., 1987; Greenberg et al., 1989; Pelzer et al., 1989; Gilly and Scheuer, 1993; Brezina et al., 1994; Yamoah and Crow, 1994) raise several interesting questions including those on the evolutionary origin and phylogenetic significance of such differences, on the nature of various drug receptors on ion channels, and on allosteric interactions between these receptors. It is also not clear if the Ca^{2+} channels of insect muscles show similar differences from vertebrate channels as seen in insect neurons. Of immediate importance are questions directly related to the nature, diversity and pharmacological sensitivity of Ca^{2+} channels in *Drosophila* muscle. In this report we resolve the Ca^{2+} channel current from *Drosophila* muscles into its components and study the pharmacological sensitivity of these components.

Materials and Methods

Preparation. Calcium channel currents of larval body-wall muscles (Jan and Jan, 1976; Wu and Haugland, 1985) were studied using the two-microelectrode voltage-clamp technique. Wandering third instar larvae (Wu and Haugland, 1985; Singh and Wu, 1989) of the wild-type *Drosophila melanogaster* strain Canton-S were used. Flies were grown on a standard cornmeal medium at 21°C. The larvae were pinned dorsal side up on a dissection dish and placed under a dissection saline. The cuticle along the dorsal midline was cut and pinned back. All internal organs were removed, allowing a clear preparation of body-wall muscle fibers. Current recordings were obtained from ventro-lateral longitudinal muscle fibers 7, 6, 13, and 12, from abdominal segments 2–6, excluding cells that endured obvious physical damage.

Electrophysiology. Electrodes were pulled from thin walled, 1.0 mm, borosilicate glass capillaries with filaments (World Precision Instruments, Sarasota, FL.) using a David Kopf Instruments puller, model 750. The voltage electrode was filled with 2.5 M KCl and the current electrode with a 3:1 mixture of 2.5 M KCl:2 M potassium citrate (Singh and Wu, 1989). Resistance of both electrodes was in the range of 10–20 MΩ.

Currents were elicited by 500 msec voltage steps, from a holding potential of −100 mV or −30 mV (as stated), to potentials between −60 and +40 mV, in 10 mV increments. Pulses were given at 10 sec intervals. No Na^+ current is observed in *Drosophila* muscles (Salkoff and Wyman, 1983; Wu and Haugland, 1985). In general, there has been no direct demonstration of Na^+ current in insect muscles (Pichon and Ashcroft 1985; Hille, 1992). K^+ currents were blocked by tetraethylammonium, 4-aminopyridine, and quinidine (Wu and Haugland, 1985; Gho and Mallart, 1986; Singh and Wu, 1989). In experiments with 1,4-dihydropyridines, a holding potential of −30 mV was used, as the effects of these agents are voltage dependent. A slight run-down of current amplitude was observed, but was not significant in early recordings. Therefore, all recordings were made within thirty minutes after the dissection began. The temperature of the recording chamber was maintained at 4°C with a peltier junction and measured for each larva with a thermocouple probe.

Solutions. The dissection saline contained (in mM): 128 NaCl, 35.5 sucrose, 2 KCl, 5 HEPES, and 4 $MgCl_2$ at pH 7.1. The current recorded in all experiments was barium current through Ca^{2+} channels in a Ca^{2+} free solution. A Ca^{2+} free solution helps prevent muscle contraction during recording, prevent activation of the calcium-dependent potassium channels, and prevent calcium-dependent inactivation of the Ca^{2+} channels. The recording saline contained (in mM): 128 NaCl, 35.5 sucrose, 2 KCl, 5 HEPES, 20 tetraethylammonium, 1 4-aminopyridine, 10 $BaCl_2$, 4 $MgCl_2$, and 0.1 quinidine at pH 7.1.

Drugs. (±)Verapamil hydrochloride, diltiazem hydrochloride, amiloride hydrochloride, and nifedipine were obtained from Sigma Chemical Company, St. Louis, MO. Isradipine (PN200-110) and (±)Bay K 8644, generous gifts from Dr. David Triggle (SUNY Buffalo), came respectively from Sandoz (Basel, Switzerland) and Miles Pharmaceuticals (West Haven, CT). All drugs were stored at −12°C in the dark, diluted to their respective desired concentrations immediately before each experiment, and used under minimal light conditions. Dihydropyridines were dissolved in ethanol. The maximum final ethanol concentration used in the experiments was 0.2%. This concentration was without effect on the barium current of control fibers.

Equipment and software. A Macintosh IISi provided the voltage-clamp command pulses through a 12 bit digital-to-analog converter using a MacADIOS II/16 board from GW Instruments (Somerville, MA). An Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) or a Turbo TEC 01C/02/03 two-electrode clamp (npi electronic GmbH, Tamm, Germany) was used for recordings. Data were acquired after 16 bit analog-to-digital conversion. Further analysis was performed with a program written in Think-C (Symantec Corporation, Cupertino, CA).

Data handling. The test currents were digitally sampled every 500 μsec, except during examination of capacitive transients (Wu and Haugland, 1985), which were sampled every 100 μsec. Currents were digitally corrected for linear leakage with respect to currents obtained at −60 mV. Current densities, expressed as nanoamperes per nanofarad (nA/nF), were calculated by dividing the measured current by the capacitance of the cell to avoid differences due to fiber size. All traces show the average of data obtained from a number of fibers as denoted. Current amplitude for each pulse was measured at the peak. The inhibition reported for various pharmacological agents was determined by comparing the peak current amplitude obtained at 0 mV. Data are expressed as mean values ± standard errors of mean (SEM).

Results

Whole-cell calcium channel currents

The current recorded with a holding potential of −100 mV consisted of a transient phase reaching its peak rapidly, and a more sustained phase that persisted with maintained depolarization (Fig. 1*A*); 100 μM Cd^{2+} blocked the current, signifying it to be Ca^{2+} channel current (Fig. 1*B*). The current–voltage (*I/V*) relationship for the current is shown in Figure 1*C*. The maximum current, observed at 0 mV, reached a peak of −18.04 ± 1.07 nA/nF (n = 15).

It is likely that the total Ca^{2+} channel current seen in Figure 1 might consist of more than one component. One effective method to resolve ionic currents uses differences in the voltage range of activation (Fox et al., 1987; Hille, 1992). For example, the T-type current in vertebrate preparations generally activates at potentials positive to about −70 mV membrane potential and dominates at potentials negative to −20 mV. The vertebrate L-type current on the other hand generally activates at membrane potentials positive to about −20 mV. *Drosophila* muscles did not show any Ca^{2+} current at potentials negative to −30 mV (Fig. 1*C*). These results are similar to those from *Drosophila* embryonic neurons where multiple types of whole-cell calcium currents all activate at potentials above −40 mV (Byerly and Leung, 1988). Similarly, all subtypes of single Ca^{2+} channels in *Drosophila* neurons activate within the same range of potentials and can not be classified by their activation threshold (Leung and Byerly, 1991). If *Drosophila* muscles also have more than one type of Ca^{2+} channel with similar thresholds of activation, it would be difficult to distinguish them on such a basis. We therefore relied on pharmacological agents and on differences in inactivation properties to resolve individual currents.

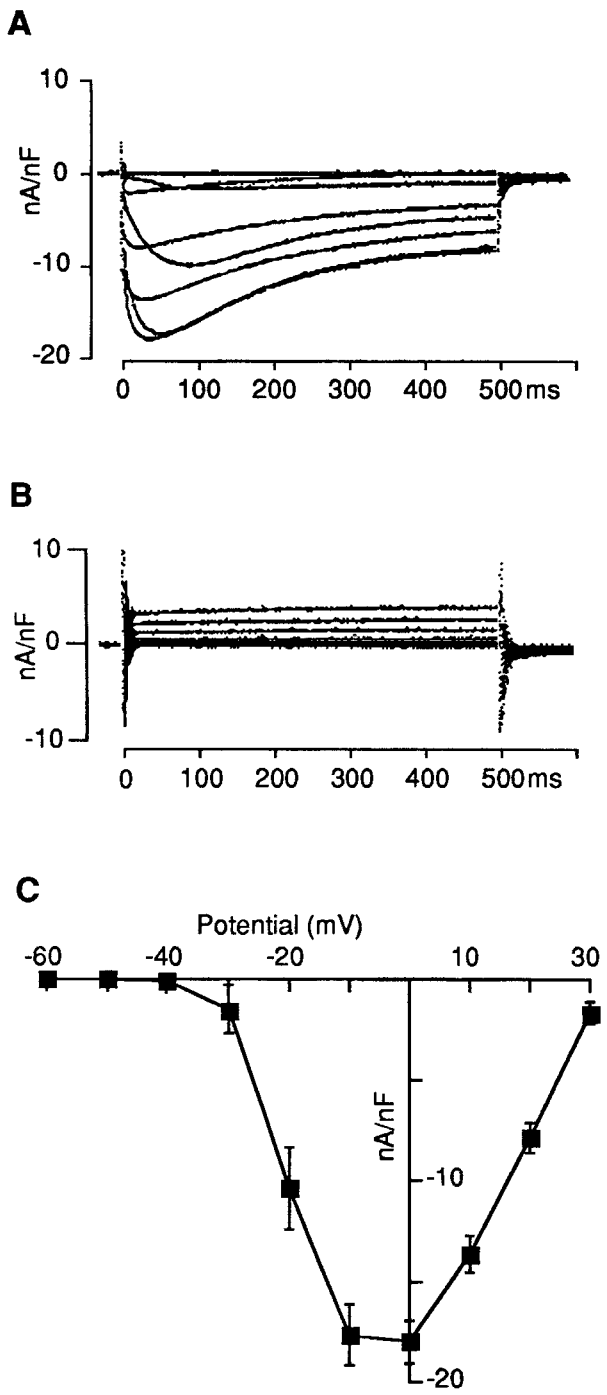


Figure 1. Ca^{2+} channel currents from larval muscle fibers of *Drosophila* elicited from a holding potential of −100 mV. *A*, Traces show leak-subtracted membrane current recordings obtained by 500 msec voltage-clamp pulses from −60 mV through +30 mV in 10 mV increments. Unless otherwise mentioned, current traces in all subsequent figures were obtained with the same pulse protocol. *L* (number of larvae) = 10; *F* (number of fibers) = 15. *B*, Blockade of calcium channel currents by 100 μM cadmium (*L* = 3; *F* = 9). *C*, The current–voltage relationship (*I/V*) for the peak value of the current. Currents shown in this and subsequent figures were normalized to membrane capacitance to avoid differences due to fiber size.

An amiloride sensitive component of the Ca^{2+} channel current

Since the T-type current is prevalent in vertebrate muscles, we wanted to check if a corresponding current contributed to the total Ca^{2+} channel current in *Drosophila* muscles. A few agents

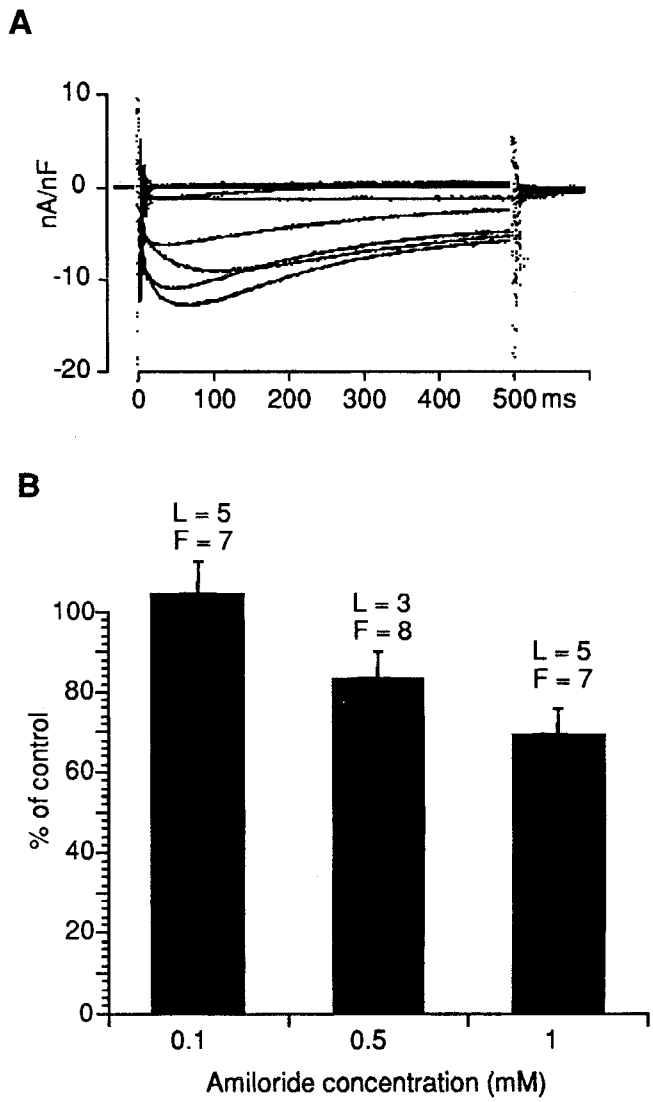
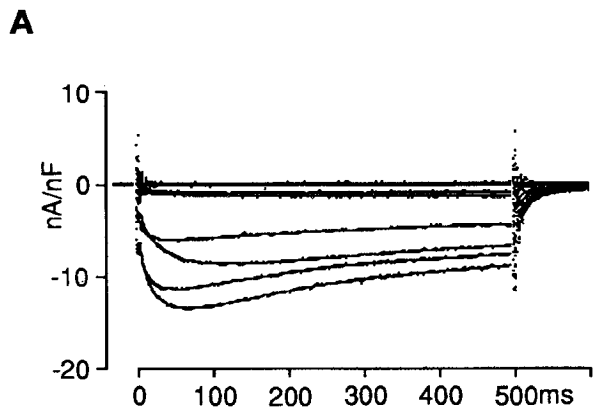


Figure 2. Effect of amiloride on the Ca^{2+} current. *A*, Current elicited from a holding potential of −100 mV in the presence of 1 mM amiloride. *L* = 5; *F* = 7. *B*, Blockade of the peak current (at 0 mV) by increasing concentrations of amiloride. At 1 mM, the peak current was inhibited by 31.6 ± 6.1%.

inhibit T-type channels, although their effects are generally not specific (Tytgat et al., 1988; Llinas et al., 1989). Amiloride is the most selective of the known T-type channel blockers and has been most extensively used. It selectively blocks the T-type channels in mouse neuroblastoma, chick DRG neurons (Tang et al., 1988), canine cardiac Purkinje cells (Hirano et al., 1989) and guinea pig ventricular myocytes (Tytgat et al., 1990). In these studies, approximately 1 mM amiloride produces near total blockade, although incomplete block can be achieved with lower concentrations. Figure 2*A* shows the currents obtained from *Drosophila* muscles in the presence of 1 mM amiloride. On average, 1 mM amiloride reduced the peak current at 0 mV by 31.1 ± 6.1% (n = 7). A part of the total Ca^{2+} channel current in *Drosophila* muscle fibers is thus similar to the vertebrate T-type current in its sensitivity to amiloride. Figure 2*B* represents the peak current inhibition with various concentrations of amiloride.

In vertebrates, T-type Ca^{2+} channels are virtually inactivated at a depolarized holding potential (V_h) such as −30 mV, while L-type channels are not (Fox et al., 1987). Recordings with −30



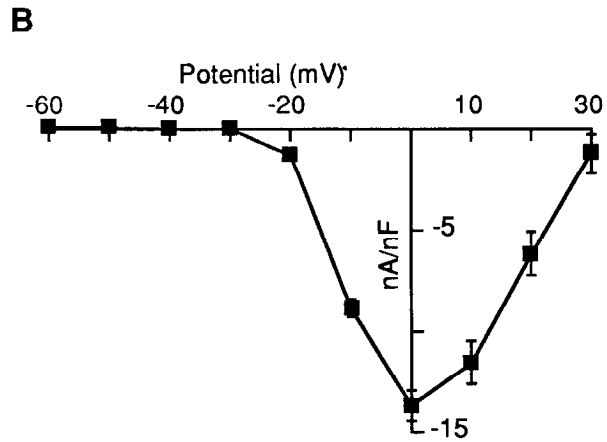


Figure 3. A depolarized holding potential inactivates part of the current. *A*, Ca^{2+} channel currents from larval muscle fibers of *Drosophila* elicited from a depolarized holding potential of −30 mV. *B*, The current–voltage relationship for currents shown in *A*. $L = 9$; $F = 12$.

mV V_h were pursued to examine if Ca^{2+} current in *Drosophila* inactivated at this V_h. Figure 3 shows the currents obtained and the corresponding *I/V* plot. Holding the membrane at −30 mV decreased the peak current by 23.8 ± 7.3% ($n = 12$) compared to −100 mV.

It would be interesting to see if the current component inactivated by −30 mV V_h corresponds either completely or partly to the amiloride-sensitive current. To address this question, we recorded the currents from a holding potential of −30 mV in the presence of 1 mM amiloride. If amiloride and depolarized holding potential affect two different components of the total current, amiloride is expected to further reduce the current recorded with a V_h of −30 mV. Amiloride did not show such an effect (Fig. 4). This indicates that it is likely to be the amiloride-sensitive current that is inactivated at −30 mV. This also indicates that amiloride may act selectively on the current which is inactivated at −30 mV V_h without blocking the noninactivating current.

A dihydropyridine sensitive component of the Ca^{2+} channel current

An L-type current, sensitive to 1,4-dihydropyridines (DHPs), forms an important component of many vertebrate cells. To examine if a DHP-sensitive current existed in *Drosophila* muscle

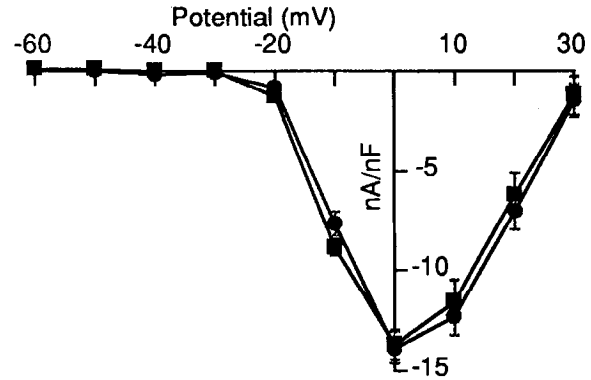


Figure 4. Current–voltage relationship of the effect of amiloride on currents elicited from a holding potential of −30 mV. Both control currents (■: $L = 9$; $F = 12$) and currents in the presence of 1 mM amiloride (●: $L = 3$; $F = 7$) were obtained with a holding potential of −30 mV.

fibers, we tested the effect of a DHP antagonist PN200-110. In voltage-clamp experiments, the ability of DHP antagonists to block Ca^{2+} current is strongly affected by holding potential (Bean, 1984; Sanguinetti and Kass, 1984; Uehara and Hume, 1985). The binding affinity of DHP antagonists increases dramatically in a variety of systems upon membrane depolarization (Kokubun et al., 1986; Greenberg et al., 1989; Wei et al., 1989). The modulated-receptor hypothesis (Hille, 1977) proposes that the affinity of a ligand for its receptor depends on the channel state, which may change with the membrane potential. Depolarization promotes the inactive channel state, increasing the potency of DHP antagonists (Bean, 1984). Therefore, all experiments with PN200-110 were performed from a holding potential (V_h) of −30 mV, encouraging high affinity binding to its receptor. Note that with a V_h of −30 mV, the amiloride-sensitive current is already inactivated. At this V_h, PN200-110 produced near complete blockade of the current (Fig. 5*A*), with 10 μM PN200-110 giving 92.6 ± 6.4% blockade ($n = 9$). Experiments with a V_h of −100 mV showed relatively little channel blockade with similar concentrations (data not shown), indicating a voltage dependence of the effects of PN200-110. Figure 5*B* represents the peak current inhibition seen with various concentrations of PN200-110. It also shows the blockade of the current by 10 μM nifedipine and 10 μM nitrendipine, two other frequently used dihydropyridine antagonists of the vertebrate L-type current. The data indicate that 50% current inhibition would require between 1 and 5 μM PN200-110. DHP concentrations between 1–10 μM produce L-type Ca^{2+} channel blockade in frog skeletal muscle (Palade and Almers, 1985; Neuhaus et al., 1990), chick sensory neurons (Fox et al., 1987), guinea pig cardiac cells (Lee and Tsien, 1983; Hess et al., 1984), and human skeletal muscle fibers (Garcia et al., 1992). Since micromolar concentrations of DHP antagonists can sometimes block potassium currents (DeCoursey et al., 1985), another important criterion for identifying vertebrate L-type channels is the action of dihydropyridine agonists such as Bay K 8644. The DHP-sensitive current in *Drosophila* muscles showed a dramatic increase in the tail currents in response to Bay K 8644 (Fig. 5*C*). These data suggest that the current elicited from a holding potential of −30 mV may be mediated through Ca^{2+} channels which are sensitive to 1,4-dihydropyridines.

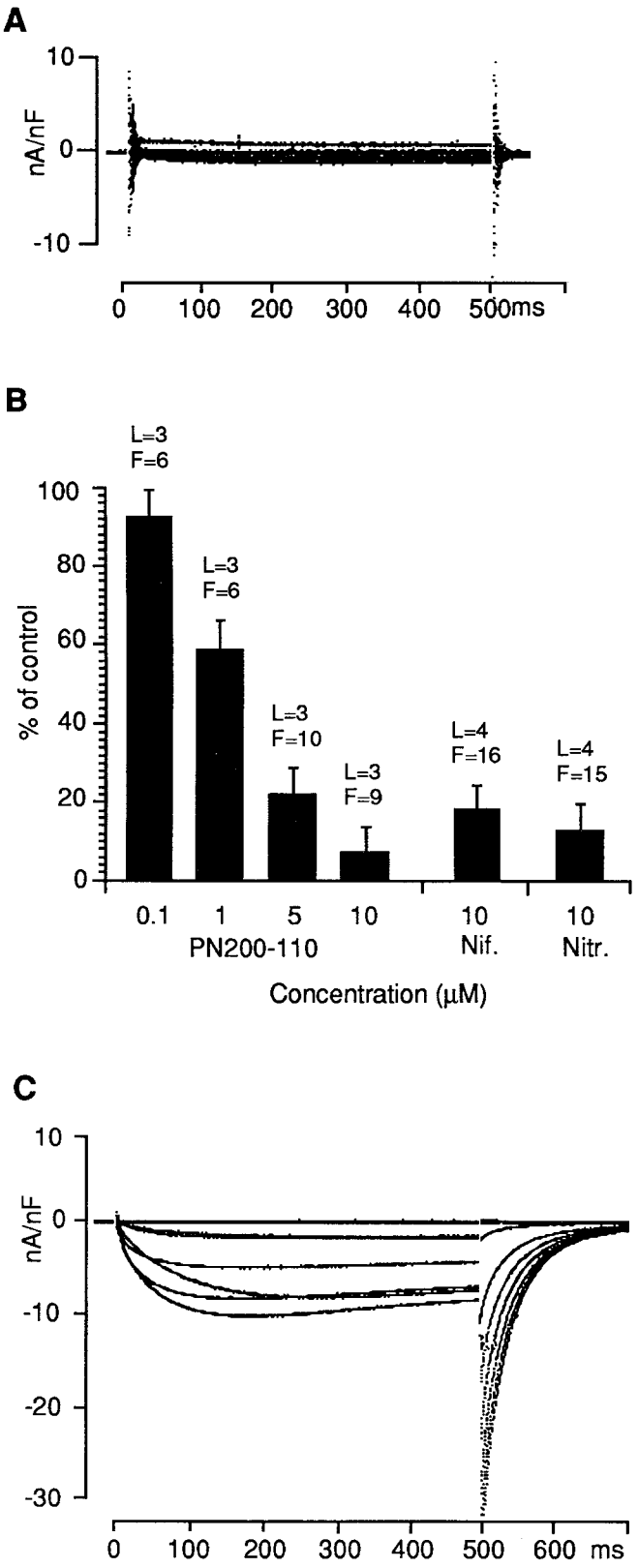


Figure 5. 1,4-Dihydropyridine sensitivity of one component of the Ca^{2+} current. *A*, Currents recorded from a holding potential of −30 mV in the presence of 10 μM PN200-110. $L = 3$; $F = 9$. *B*, Blockade of the peak current (at 0 mV) by increasing concentrations of PN200-110; by 10 μM nifedipine (*Nif.*); and by 10 μM nitrendipine (*Nitr.*). At 10 μM PN200-110, the peak current was inhibited by 92.6 ± 6.4%. *C*, Enhancement of tail currents (compare with the tail currents in Fig. 3*A*) by 1.0 μM Bay K 8644 in recordings made from a holding potential of −30 mV ($L = 4$; $F = 14$).

Effect of verapamil and diltiazem on the 1,4-dihydropyridine-sensitive current

Since the vertebrate L-type channels are sensitive to DHPs, phenylalkylamines (e.g., verapamil) as well as benzothiazepines (e.g., diltiazem) (Triggle, 1990) whereas a majority of Ca^{2+} channels from *Drosophila* brain membranes are sensitive to verapamil but not to DHPs (Pelzer et al., 1989), it would be very interesting to see if the DHP-sensitive channels from *Drosophila* muscle are sensitive to verapamil and diltiazem. We examined the effect of these two agents at a holding potential of −30 mV which isolates the DHP-sensitive current from the amiloride-sensitive component.

High concentrations of verapamil were required to block the DHP-sensitive current in *Drosophila* muscles (Fig. 6*A*). Figure 6*B* shows the effect of increasing concentrations of verapamil, with 96.2 ± 7.4% ($n = 12$) blockade occurring at 500 μM verapamil. The data indicate that 50% blockade would require between 10 and 100 μM verapamil. The major fraction of single channels obtained from brain membrane preparations in *Drosophila* show a marked inhibition by 1 nM $(-)D_{600}$ (Pelzer et al., 1989); 1–15 μM concentrations of phenylalkylamines reduce the L-type channel current in guinea pig cardiac cells (Lee and Tsien, 1983) and frog skeletal muscle (Palade and Almers, 1985). This indicates that the DHP-sensitive channels in the *Drosophila* muscles may be relatively less sensitive to verapamil than the phenylalkylamine-sensitive channels from *Drosophila* brain membranes or the vertebrate L-type channels.

Diltiazem inhibited the current recorded from a holding potential of −30 mV, with 1 mM blocking about 96.0 ± 4.3% of the current ($n = 16$) (Fig. 7*A*). Figure 7*B* shows inhibition with increasing diltiazem concentrations. The data indicate that 50% inhibition would require a little over 100 μM diltiazem. For comparison, 50 μM diltiazem produces L-type current blockade in guinea pig cardiac cells (Lee and Tsien, 1983) and 500 μM diltiazem produces approximately 50% Ca^{2+} current blockade in *Xenopus* oocytes expressing rat skeletal muscle RNA (Dascal et al., 1992).

Five hundred micromolar verapamil blocked about 71.0 ± 7.9% of the total current recorded with a V_h of −100 mV ($n = 15$) (Fig. 6*C*). Similarly, 1 mM diltiazem blocked about 74.8 ± 8.0% of the total current elicited from a V_h of −100 mV ($n = 16$) (Fig. 7*C*). Since the DHP-sensitive current constitutes approximately 70–75% of the total current elicited from this holding potential (Figs. 2, 3), verapamil and diltiazem blocked most of this current, as they had done at a V_h of −30 mV, without an apparent effect on the amiloride-sensitive current. The data also indicate that the action of verapamil and diltiazem may not depend on the holding membrane potential.

If the total calcium current recorded at a V_h of −100 mV consists primarily of the DHP-sensitive and the amiloride-sensitive current, application of both 1 mM diltiazem and 1 mM amiloride should block most of this current. As expected, the two drugs together blocked about 92.1 ± 5.0% of the total current ($n = 16$) (Fig. 8).

Resolution of the dihydropyridine-sensitive and the amiloride-sensitive current

With the above observations, it becomes easy to resolve the two Ca^{2+} currents in the larval muscles. The amiloride-sensitive current can be recorded at a holding potential of −100 mV by blocking the DHP-sensitive current by diltiazem (Fig. 7*C*). DHP

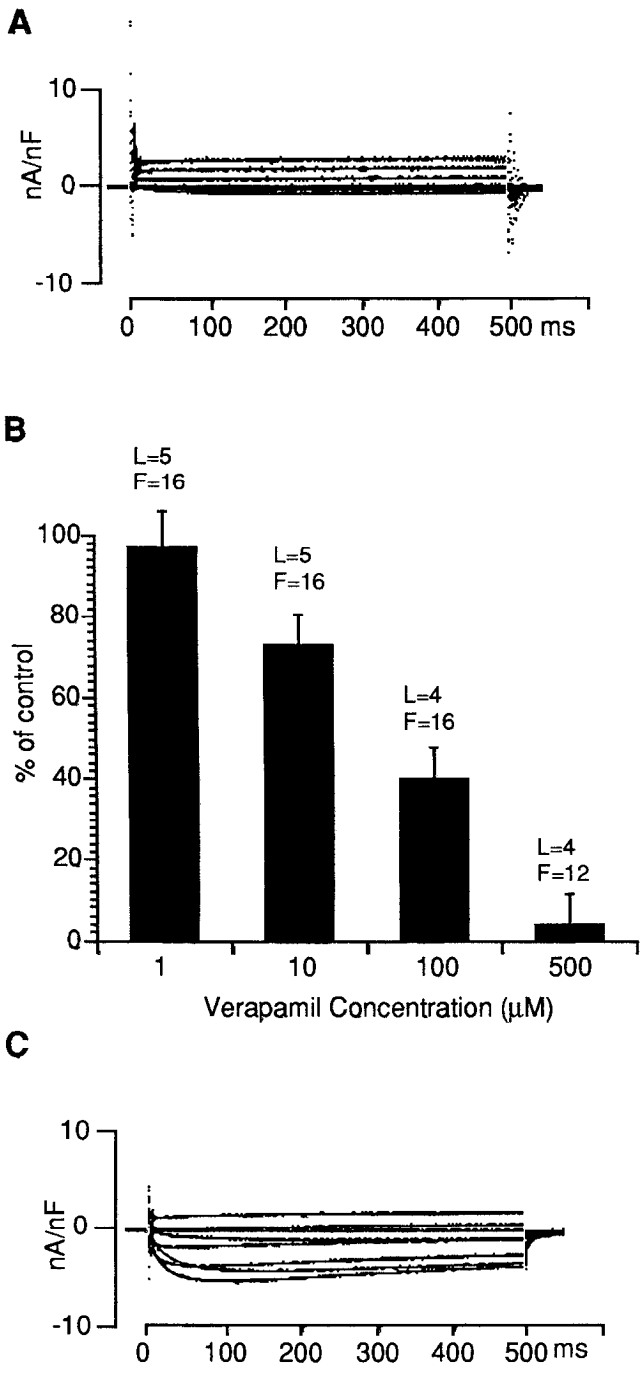


Figure 6. Effect of verapamil on the dihydropyridine-sensitive current. *A*, Currents recorded from a holding potential of −30 mV in the presence of 500 μM verapamil, with pulses up to +40 mV. *B*, Blockade of the peak current (at 0 mV) by increasing concentrations of verapamil. At 500 μM verapamil, the peak current was inhibited by 96.2 ± 7.4%. *C*, Blockade of the current recorded from a holding potential of −100 mV by 500 μM verapamil. The peak current was inhibited by about 71.0 ± 7.9%.

blockers can not be used for this purpose because they act only at depolarized potentials at which the amiloride-sensitive current is inactivated. The DHP-sensitive current can be isolated from the amiloride sensitive current either by using 1 mM amiloride (Fig. 2*A*) or by holding the membrane potential at −30 mV (Fig. 3*A*).

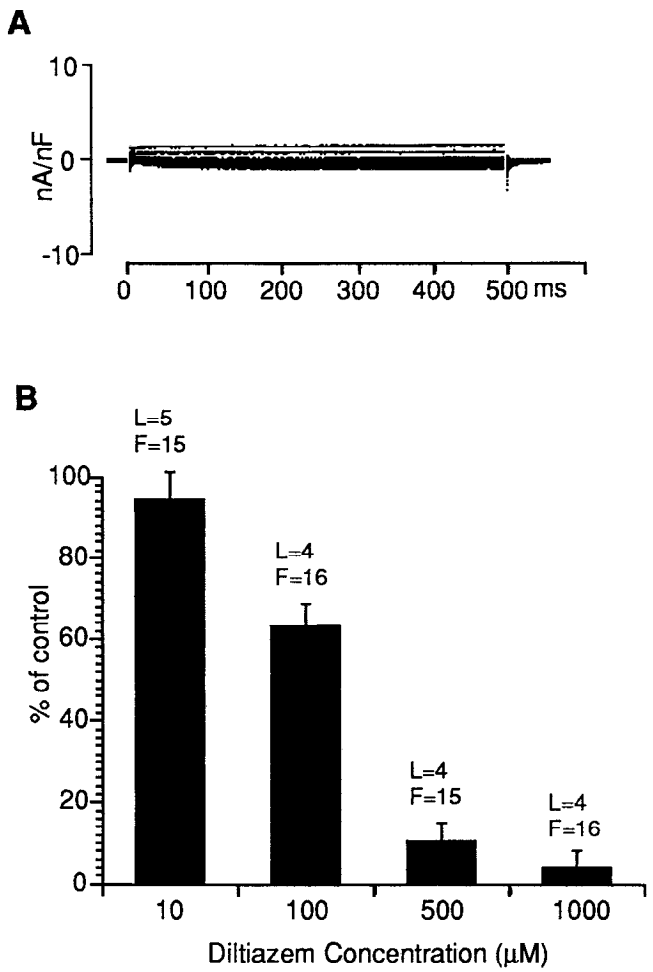
Discussion

This report describes currents through the voltage-dependent calcium channels of the body-wall muscles of *Drosophila* larvae. Measurements revealed two main components of the current, a 1,4-dihydropyridine-sensitive component and an amiloride-sensitive component.

Membrane excitability in the larval body-wall muscles of *Drosophila* is based on Ca^{2+} and K^+ channels. K^+ channels have been previously resolved into four components, namely I_A, I_K, I_{CF}, and I_{CS} (Singh and Wu 1989, Singh and Wu, 1990). Resolution of the Ca^{2+} current into two components in the present study thus provides one of the few preparations in which the whole-cell current can be completely resolved into its individual components. This separation of currents will facilitate the analysis of the physiological and pathological roles of each current, as well as greatly aid in the genetic and pharmacological analysis of the individual ion channels and their modulation. Ability to record each of the two Ca^{2+} current components in isolation from other currents would be helpful in identifying mutations that affect either of these two currents. This would in turn be very useful in undertaking a mutational analysis of the structure, function and regulation of these channels. Analysis of pharmacological and physiological differences between the insect and the vertebrate Ca^{2+} channels will be useful in designing useful strategies for the control of harmful insects.

As mentioned in the introductory section and Results, earlier literature has revealed pharmacological differences between vertebrate and insect Ca^{2+} channels and raised questions on the extent of these differences. Our data show an overlap in pharmacological specificity of the L-type and the T-type vertebrate currents with the two currents described in this report. Sensitivity to 1,4-dihydropyridines (PN200-110, nifedipine and nitrendipine) and benzothiazepines (diltiazem) is characteristic of the vertebrate L-type Ca^{2+} current. Amiloride-sensitivity is characteristic of the T-type Ca^{2+} current in vertebrates. This argues that the pharmacology of insect Ca^{2+} channels may not be entirely different from that of mammalian channels.

We did not examine detailed physiological properties of the two currents. However some simple observations can be made from the data presented here. The activation threshold of between −30 and −20 mV for the DHP-sensitive current (Fig. 3*B*) was comparable to the vertebrate L-type current which starts activating around −30 to −10 mV (Tsien et al., 1988; Hille, 1992). Almost complete inactivation of the *Drosophila* amiloride-sensitive current at a holding potential of −30 mV corresponded to inactivation of the vertebrate T-type current under similar conditions. On the other hand, as mentioned in Results, the activation threshold of −30 mV for the amiloride-sensitive current was higher than the activation threshold of about −70 to −50 mV for the vertebrate T-type current (Tsien et al., 1988; Hille, 1992). In addition, the current in Figure 7*C* does not show a transient nature characteristic of the vertebrate T-type current (Fox et al., 1987). The *Drosophila* muscle current inactivated by about 22.3 ± 11.7% (n = 16) of the peak value during a 500 msec pulse to 0 mV (calculated from Fig. 7*C*). On the other hand, the vertebrate T-type current shows rapid inactivation, with a time constant ranging between about 20 and 50 msec (Tsien et al., 1988). These difference are not surprising since physiological properties of invertebrate Ca^{2+} currents often differ from those of vertebrate Ca^{2+} currents (Strong et al., 1987; Leung and Byerly, 1991).



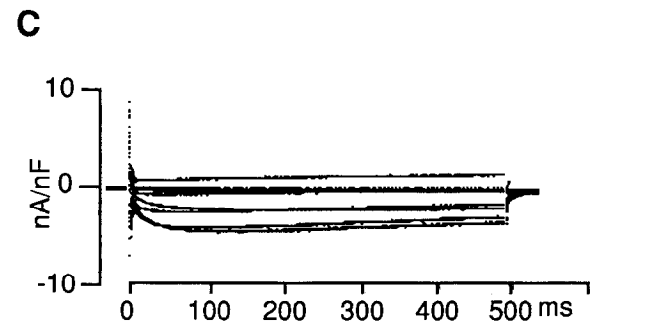


Figure 7. Blockade of the dihydropyridine-sensitive current by diltiazem. *A*, Currents recorded from a holding potential of −30 mV in the presence of 1 mM diltiazem, with pulses up to +40 mV. *B*, Blockade of the peak current (at 0 mV) by increasing concentrations of diltiazem. At 1 mM diltiazem, the peak current was inhibited by 96.0 ± 4.3%. *C*, Blockade of the Ca^{2+} current recorded from a holding potential of −100 mV by 1 mM diltiazem. The peak current was inhibited by about 74.8 ± 8.0%.

It was suggested in the Results that the current inactivated by a holding potential of −30 mV is likely to be the same as the amiloride-sensitive current. This interpretation was linked to the lack of amiloride action on the current recorded at the V_h of −30 mV. With the current experimental protocol, we cannot rule out the possibility that the action of amiloride may be voltage dependent such that it may not act at a depolarized V_h of −30 mV.

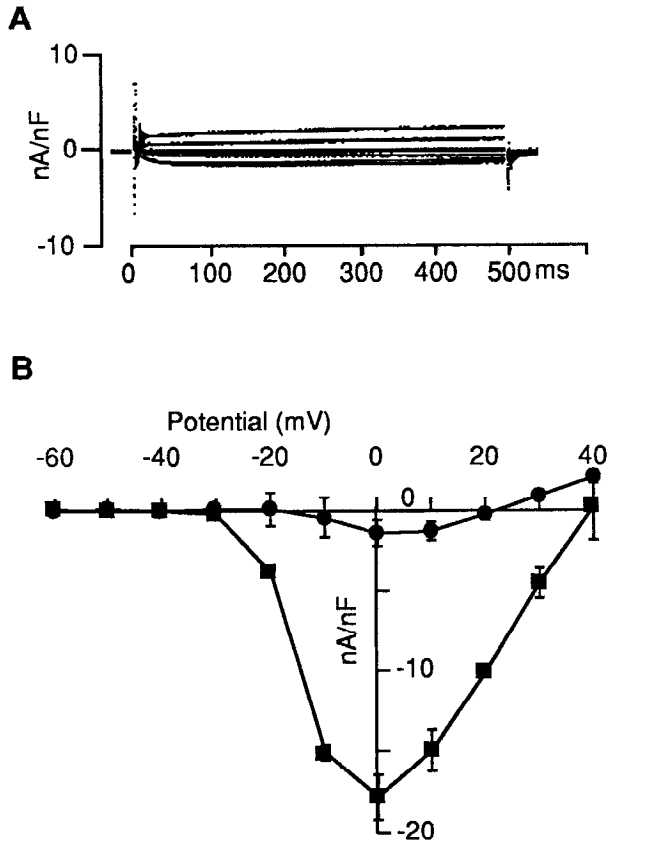


Figure 8. Effect of diltiazem and amiloride together on the total Ca^{2+} channel current. *A*, Currents recorded from a holding potential of −100 mV in the presence of 1 mM diltiazem and 1 mM amiloride, with pulses up to +40 mV. *B*, Current–voltage relationship for the currents recorded in the presence (●: $L = 4$; $F = 16$) or absence (■: $L = 5$; $F = 17$) of 1 mM diltiazem plus 1 mM amiloride.

However, another line of argument supports the likely overlap between the amiloride-sensitive current and the current inactivated at a V_h of −30 mV. Experiments with diltiazem and amiloride indicate that the total Ca^{2+} current recorded from a V_h of −100 mV consists primarily of the DHP-sensitive and the amiloride sensitive components (Fig. 8). An almost complete blockade of the noninactivating component at a holding potential of −30 mV by diltiazem argues that the amiloride-sensitive current has likely been inactivated at this holding potential.

Our data raise several interesting questions on the pharmacology of the dihydropyridine/phenylalkylamine-sensitive Ca^{2+} channels. Previous single-channel studies on brain membrane preparations of *Drosophila* show that most channels are sensitive to phenylalkylamines but insensitive to DHPs, while a small fraction are sensitive to DHPs, but insensitive to phenylalkylamines (Pelzer et al., 1989). Thus, a particular channel molecule is proposed to have a binding site for either verapamil or DHPs, but not both. This is in contrast to the vertebrate L-type channels which not only bind DHPs, phenylalkylamines and benzothiazepines on the same subunit, but also show allosteric interactions between the three binding sites (for review, see Catterall et al., 1988; Hosey and Lazdunski, 1988; Glossmann and Striessnig, 1990; Triggle, 1990). The major component of the whole-cell Ca^{2+} current from *Drosophila* muscles, in the present study, was blocked by PN200-110 and diltiazem but showed low sensitivity to verapamil. It will be interesting to examine the pharmacology

of single channels from these muscles and ask if individual channels have receptors for both DHPs and benzothiazepines and if these receptors show allosteric interactions as they do in vertebrate L-type channels. It will also be interesting to see if diltiazem affects either of the two *Drosophila* neuronal Ca^{2+} channel subtypes, the ones that show DHP sensitivity and the ones that show phenylalkylamine sensitivity (Pelzer et al., 1989). Distribution of various subtypes of dihydropyridine/phenylalkylamine-sensitive Ca^{2+} channels appear tissue specific, with neurons showing predominantly phenylalkylamine-sensitive channels and muscles showing a predominantly 1,4-dihydropyridine-sensitive current. With a rapid increase in our knowledge of the diversity of various subtypes of channels and their tissue-specific expression, it will be interesting to see if all muscles express one subtype and all neurons another subtype of channels, or if there are differences among various muscles or various populations of neurons. The muscle currents shown here were recorded from larvae, whereas brain membrane preparations in previous reports were made from adult flies. The observed differences may also be related to a difference in the developmental stage. Differential splicing may also play some role in the diversity and the tissue-specific expression of various subtypes of currents. Another very interesting aspect to examine would be the evolutionary relationship between the vertebrate and the insect Ca^{2+} channels on one hand, and between the neuronal and the muscle Ca^{2+} channels in *Drosophila* on the other. Further studies, some involving single-channel analysis, are needed to answer many of these questions.

All recordings in our experiments were made within thirty minutes of the start of the dissection. The currents displayed evidence of "rundown" with time after the dissection, but current reduction was quite minimal within the first 30 min. Rundown of Ca^{2+} currents is commonly observed during whole-cell patch-clamp recordings, and is even more pronounced with excised patches. This occurs as small molecules important for maintenance of channel function are eluted from the cytoplasm. Reduction in phosphorylation of the channel protein may also be involved (Byerly and Hagiwara, 1982; Fenwick et al., 1982; Byerly and Leung, 1988; Leung et al., 1989). An explanation of a run-down is less clear in our experiments, as it occurs over time even while the cells are intact. Procedures allowing good current recordings, including the dissection and the presence of barium, may eventually damage the tissue resulting in metabolically compromised cells and subsequent rundown of the Ca^{2+} channel currents.

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